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13. ABSTRACT (Maximum 200 Words)

The goal of this proposal is to study the potential role of tyrosine phosphorylation-dependent checkpoint and especially the cdc25 phosphatase family of cyclin dependent kinase (CDK) activators in cellular immortalization. We have shown that cdc25 phosphatases did in fact extend the normal mammary epithelial cells (HMEC) life span, but do not immortalize them. The effect was most prominent with cdc25A and cdc25B phosphatases. Cdc25C phosphatase had less effect on HMEC life span. Experiments with catalytically inactive cdc25A mutant show that phosphatase activity of cdc25A is essential for the life span extension. Our experiments show that telomerase is not activated by cdc25A expression in normal human cells, suggesting that cdc25 most likely affect M1, but not M2 checkpoints. Retroviral cDNA libraries (sense and anti-sense) were introduced into HMEC together with cdc25 to assay for the potential "enhancement" of cdc25 ability to expand the cellular life span. Work is underway to identify genes affected by the appropriate antisense constructs.

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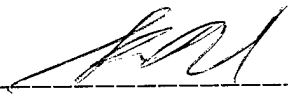
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Introduction

A variety of genetic abnormalities contribute to the transformation of normal cells into aggressively growing cancer cells. Among them, loss of the normal mechanisms of proliferation control is the common theme of most human malignancies.

The cell cycle in mammalian cells is driven by the family of evolutionary conserved cyclin-dependent kinases or CDKs (CDK1-9; Morgan, 1995). As cells progress in the cell cycle, CDKs are activated and promote further passage from one cell cycle phase to another by phosphorylation of critical substrates (reviewed in Draetta, 1990; Morgan, 1995). CDKs are inactive unless associated with the cyclin subunit. So far, eight different types of cyclins have been identified in human and other higher eukaryotes (cyclins A-H; reviewed in Hunter and Pines, 1994; Morgan, 1995; Sherr, 1996). Each CDK has a preferential cyclin subunit as a partner; however, some cyclins have the ability to associate with more than one CDK, creating a large number of cyclin/kinase complexes (Morgan, 1995; Sherr, 1996). Different cyclin-CDK complexes are activated at distinct points in the cell cycle (reviewed in Sherr, 1993,1996; Sherr and Roberts, 1995; Weinberg, 1995). In addition, mammalian CDKs are negatively regulated by the diverse family of cyclin-dependent kinase inhibitors or CKIs: p16ink4/MTS1, p15ink4b/MTS2, p21cip1/WAF1/SDI1(a transcriptional target of p53), p27kip1, p57kip2 and others (reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996). These molecules specifically target CDKs important for G1-S transition: CDK2, CDK4 and CDK6. Finally, CDKs are regulated by positive (CAK, or CDK activating kinase) or negative (wee1/mik1/myt1) phosphorylation. Phosphorylation by the wee1 family of kinases is reversed by the action of cdc25 phosphatases which have a unique specificity toward CDKs (reviewed in Millar and Russel, 1992; Draetta and Eckstein, 1997). The wee1/cdc25 checkpoint was originally described in fission yeast (Russell and Nurse, 1986) and later shown to represent a universal element of the cell cycle control (Russel, Moreno and Reed, 1989; Sadhu et al., 1990; Galaktionov and Beach, 1991). All CDKs (with the exception of cdk7) contain a conserved tyrosine and in the majority of CDKs, a neighboring threonine residue, targeted by the wee1 family of kinases (Gu et al., 1992; Parker et al., 1992; Mueller et al., 1995; Terada et al., 1995; Iavarone and Massague, 1997). Phosphorylation of a kinase on either or both of these residues renders it completely inactive. In human cells, there are three known cdk-activating phosphatases, cdc25A, B and C (Sadhu et al., 1990; Galaktionov and Beach, 1991).

Transformation of the normal human cell into an aggressively growing tumor cell typically requires a series of loss of function mutations in tumor supressor genes and gain of function mutations in oncogenes. Gain of function by gene amplification has been described for c-myc and cyclin D1 in several human tumors, including breast cancer. Cyclin D1 amplification is detected in 13% of breast cancers but more than 50% appear to overexpress the protein (Sherr, 1996). Targeted overexpression of cyclin D1 in mammary epithelial cells leads to hyperproliferation and eventual tumor formation (Wang et al., 1994).

We have shown that cdc25A and cdc25B phosphatases are overexpressed in a significant portion of primary breast cancer (Galaktionov et al., 1995; Galaktionov, Chen and Beach, 1996). This overexpression might be explained in part by the action of c-myc, which is amplified in 20-30% of breast cancers, and functions in part as a transcription factor for cdc25A (Galaktionov, Chen and Beach, ibid.). Overexpression of cdc25A and cdc25B is now described in other types of human cancer as well (Gasparotto et al., 1997). Ectopic expression of cdc25A in TGFß-sensitive breast cancer cell lines renders them insensitive to the inhibitory action of this cytokine (Iavarone and Massague, 1997). As a result, cells constitutively overexpressing cdc25 phosphatases might be partially or completely insensitive to the action of TGFß, especially when the p15ink4b/mts2 (CDK4/CDK6 inhibitor, activated by TGFb) locus is inactivated by deletion. Inability of cells overexpressing cdc25 to arrest in response to negative regulatory stimuli might contribute to abnormal cell proliferation and eventually tumor formation.

The broad aim of this proposal is to investigate how perturbation of the CDK tyrosine phosphorylation checkpoint might contribute to the oncogenic transformation process.

Body

Cdc25A and cell immortalization.

As proposed in Task1, we prepared recombinant retroviruses expressing each of the three human cdc25 genes: cdc25A, cdc25B and cdc25C (Sadhu *et al.*, 1990; Galaktionov *et al.*, 1991). We used pBABE-based retroviruses since these are stable retroviral vectors and we have had previous experience working with them (Galaktionov *et al.*, 1996). To show whether the phosphatase function of cdc25 is required, we also prepared a catalytically inactive cdc25A by substituting Ser for Cys in the catalytic center of cdc25A. The ensuing titers of these viruses exceed 10^5 per ml after transient transfection into BOSC or BING cells obtained from ATCC. Mammary epithelial cells (HMEC) were obtained at passage 12 from D. Beach, Cold Spring Harbor Laboratory. Using freshly prepared retroviral stocks we were able to achieve infection rates between 10 and 40 percent routinely. This enabled us to use large infected populations without relying on single cell clones in our analysis of the potential role of cdc25 phosphatases as immortalizing agents. Infected HMECs were adjusted to the same cell count after drug selection, and growth was continued with periodic cell culture split. HMECs have a finite life span and usually exhaust their proliferation ability at about passage 20. We infected HMECs at passage 14 and counted ensuing population doublings (PDL). Control HMECs underwent only 4-5 PDLs from the point of infection before they arrested with the typical senescent cell morphology. In contrast, cells expressing cdc25A and cdc25B phosphatases continued beyond this point for 6-8 additional population doublings to total of 12-13 (Figure 1).
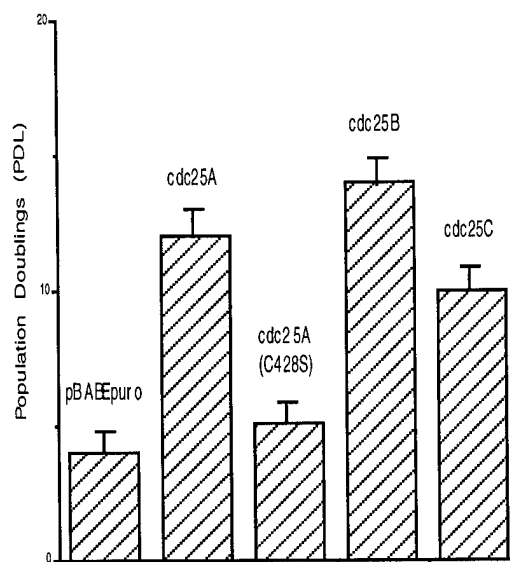

Figure 1. Life span extension by ectopic expression of cdc25 proteins. Cdc25 A, cdc25B and cdc25C, inactive cdc25A(C428S) proteins were expressed in normal human mammary epithelial cells infected with recombinant retroviruses.

We have performed these experiments three times and we are confident that cdc25 phosphatases did in fact extend the HMEC life span. The effect was most prominent with cdc25A and cdc25B phosphatases. Cdc25C phosphatase had less effect on HMEC life span. Experiments with catalytically inactive cdc25A(C482S, Figure 1) show that phosphatase activity of cdc25A is essential for the life span extension. Expression of catalytically inactive cdc25A does not cause any cell cycle arrest in HMEC, as they progress similarly to cells infected with the control retrovirus (pBABEpuro, Figure 1).

We had shown previously that a combination of oncogenic ras and cdc25A causes oncogenic transformation in rodent cells. These observations together with the experiments described here prompted us to investigate whether oncogenic rasV12 will affect the ability of cdc25A to cause life span extension in human HMEC cells. At first, we extended experiments of Serrano *et al.*, 1997, who observed that oncogenic ras causes a paradoxical reaction in normal fibroblasts, resulting in a cell cycle arrest with some evidence of the premature cell senescence (Serrano *et al., ibid.*). The cell cycle arrest caused by the ectopic expression of rasV12 requires the wild type p53 protein and is mediated at least in part by the p19ARF, encoded by the alternative reading frame of the INK4a locus (reviewed in Sherr, 1998). HMEC displays a similar phenotype as they enter the cell cycle arrest a few days after infection with pBABEpuro retrovirus expressing rasV12. The senescence phenotype was confirmed by the cell morphology (flat enlarged cells with no cell division in more than 2 weeks) and staining for the senescence-specific b-galactosidase activity (Dimri *et al.*, 1995).

Interestingly, co-expression of cdc25A or cdc25B together with rasV12 rescued HMEC cells from premature senescence caused by rasV12 (Figure 2).
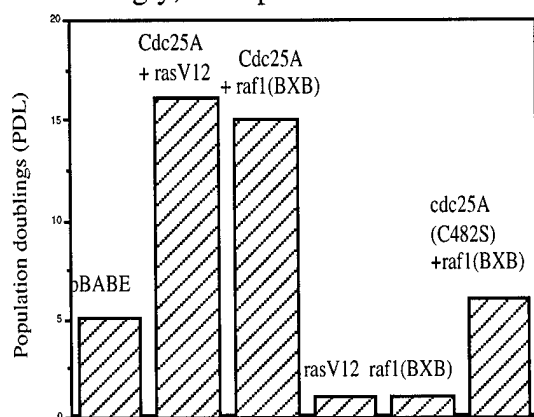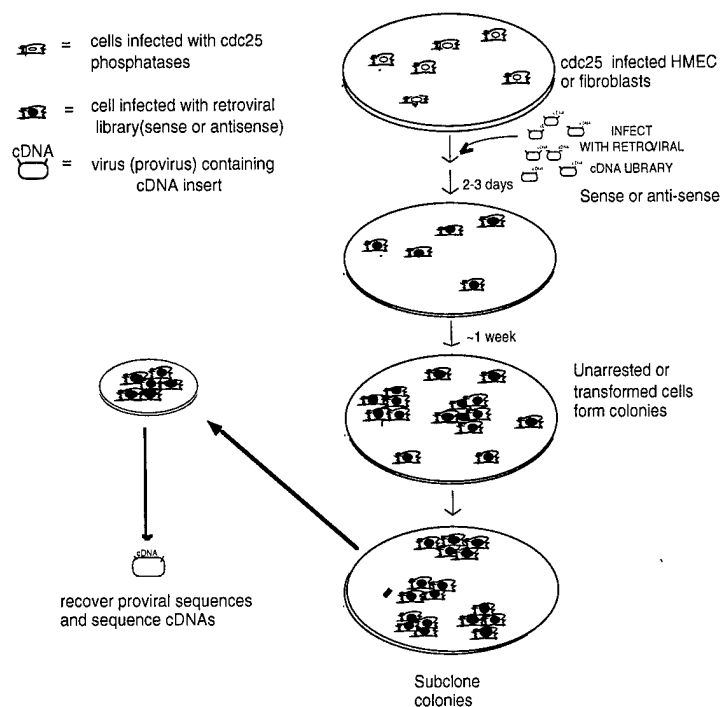

Figure 2. Life span extension by ectopic expression of cdc25A, in cooperation with rasV12 and raf1(BXB) proteins. Cdc25 A, phosphatase-"dead" cdc25A (C482S), rasV12, and raf1(BXB) proteins were expressed in normal human mammary epithelial cells infected with recombinant retroviruses. Note that rasV12 and raf1(BXB) cause a premature onset of senescence.

Because the raf/MEK/MAP kinase signaling cascade is a key effector of signaling from ras proteins, we examined the ability of raf1 kinase to affect HMEC cells. We observed that constitutively active raf1 kinase (BXB) was able to elicit cell cycle arrest and senescence (Figure 2). We wished to test whether cdc25A can reverse this arrest by co-expressing constitutively active raf1 and cdc25A. We observed that cell senescence caused by raf1 in HMEC cells was reversed by the action of cdc25A protein (Figure 2). Cells expressing both cdc25A and raf1 progress further in their life span than cdc25A alone. Interestingly, our experiments also shown that the cdc25A reversal of the cell cycle arrest caused by raf1 does not require the phosphatase activity of cdc25A, but the subsequent bypass of the normal life span does (Figure 2).

Activation of telomerase activity is often associated with the so-called M2 checkpoint, positively correlating with cell immortalization. A product of the oncogenic papilloma virus, E6, has been shown to activate telomerase activity but does not immortalize normal human mammary epithelial cells (Klingelhutz *et al.*, 1996). **As proposed in Task1,**we investigated whether this extension of the HMEC life span by cdc25 is associated with TGFβ resistance and activation of telomerase activity. To measure telomerase activity we used telomeric repeat amplification protocol (TRAP; Kim and Wu, 1997). As little as 1% telomerase-positive cells were detected by this method in a population of HMECs infected with retrovirus expressing the E6 gene product (Klingelhutz *et al., ibid.*). It has been shown recently that telomerase activity was not essential for establishment of immortal cell lines and growth of tumors in mice (Blasco *et al.*, 1997), therefore we do not necessarily expect that presence or absence of telomerase activity will represent a conclusion for our experiments, but rather another measurable parameter in addition to PDLs. The recent literature (Bodnar *et al.*, 1998), however, suggests that, in some types of human cells, ectopic expression of the catalytic telomerase subunit caused significant extension of the life span. It is possible that the contradiction between data obtained in mouse and human cells is due to species specificity and that telomerase activation is required for immortalization of the human cells. Our experiments shows that telomerase is not activated by cdc25a expression in normal human cells.

As proposed in Task1, we prepared sense and antisense cDNA libraries from polyA+ RNA isolated from growing HMEC cells. Libraries were prepared in a modified pBAbe an pWZL retroviral vectors. We began to introduce these libraries into HMEC cells that were previously infected with cdc25 expressing vectors. The overall scheme of our expreriment is shown below.

Legend text:
- = cells infected with cdc25 phosphatases
- = cell infected with retroviral library(sense or antisense)
- cDNA = virus (provirus) containing cDNA insert

cdc25 infected HMEC or fibroblasts

INFECT WITH RETROVIRAL cDNA LIBRARY

Sense or anti-sense

2-3 days

~1 week

Unarrested or transformed cells form colonies

recover proviral sequences and sequence cDNAs

Subclone colonies

To date we isolated approximately 15 independent clones of HMEC cells where we observed the 'enhancement' of cdc25-caused expansion of life span using retroviral cDNA libraries. We are currently in the process of further characterizing these clones and attempting to identify the target genes that were either affected by antisense CDNA expression (9 clones) or express sense cDNAs (6 clones). We are planning to test whether trget genes require cdc25 for their function to further extend HMEC life span or they could function independently.

2. Role of wee1 kinases in establishment of the cellular senescence program

As proposed in task2, we performed low stringency PCR using degenerate oligonucleotides in order to clone putative wee1 homolog(s) with specificity toward cdk4 and cdk6 kinases. As a result, we obtain one sequence with significant sequence homology with known wee1 gene family members. However, this sequence seems to be expressed at very low levels in a majority of human tissues and cell lines and we are currently pursuing its cloning from several cDNA libraries. Low representation of this clone so far prevented us from isolating a full length clone. **As proposed in task2,** we performed a two-hybrid screen using modified cdk4 protein. We are currently analysing cDNAs obtained in that screen by sequencing. So far, we have not identified a putative wee1 family member. **As proposed in task2,** we also started an attempt of biochemical purification of wee1 kinase, specific for cdk4. To that extent we have prepared a cellular extract from Hela cells and are currently characterising fractions obtained by anion exchange chromatography on FPLC trying first to identify the appropriate activity. The result of our biochemical experiments was that all fractions with kinase activity directed toward cdk2 had also activity directed toward cdk4 in vitro. We did not observe any fractions that had activity specifically directed toward cdk4. In addition, depletion of active fractions with antibodies against wee1 and myt1 eliminated all kinase activity against cdk2 and cdk4, suggesting that human cells does not have an additional wee1 homolog.

KEY RESEARCH ACCOMPLISHMENTS
-cdc25 extend the life span of normal breast epithelial cells (HMEC)
-cdc25 cooperate with ras in further extending the lifespan of HMEC
-we prepared sense and antisense cDNA libraries from HMEC and introduced them into HMEC. So far, we isolated 15 independent immortal clones using these libraries

REPORTABLE OUTCOMES

Abstract presented at Cell cycle and Cancer ISREC symposium, 1999, Lausanne, Switzerland
Abstract presented at Cell cycle meeting. Cold Spring Harbor, NY, 2000.
R01 grant "The cdc25 checkpoint in G1 and S phase" applied for based on findings supported by this award.

CONCLUSIONS

1. Cdc25 phosphatases expand the life span of normal mammary epithelial cells (HMEC).
2. Cdc25 phoshatases did not cause complete immortalization of HMEC
3. Phosphatase activity of cdc25A is required for the observed phenotype.
4. Cdc25 did not cause activation of telomerase in HMEC.
5. Cdc25A cooperated with RasVal12 to further expand their lifespan and reverse "senescent" phenotype caused by RasV12 in HMEC.
6. Experiments with retroviral libraries aimed at elucidating role of additional genes that might function as "enhancers" of the observed phenotypes are currently underway.
7. The two hybrid screen with "modified" cdk4 did not yield a novel wee1 gene to date.

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